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**FOR THE RECORD**

# Crystallization and preliminary X-ray investigation of lipoxygenase-3 from soybeans

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**Abstract:** Soybean lipoxygenase-3 has been crystallized by the vapor diffusion method in 16–20% polyethylene glycol (average $M_r$, 3,400), 0.2 M sodium acetate buffer, pH 5.7, at 21 °C, at a protein concentration of 8–15 mg/mL. The crystals, which diffract to 3-Å spacings, belong to the monoclinic space group C2. Cell constants are $a = 111.9$, $b = 136.4$, and $c = 61.6$ Å and $\beta = 95.7°$. The calculated value of Matthews's constant, $V_m$ = 2.48 Å$^3$/kDa, is consistent with the presence of one molecule of lipoxygenase per crystallographic asymmetric unit ($Z = 4$).

**Keywords:** lipoxygenase; lipoxygenase crystals; L-3; protein crystals; soybean

Lipoxygenases are a class of non-heme, non-sulfur iron enzymes widely distributed among plants and animals, that catalyze the dioxygenation of lipids containing a 1*Z*,4*Z*-pentadiene moiety (for recent reviews, see Gardner [1991], Siedow [1991], Yamamoto [1992], and Ford-Hutchinson et al. [1994]). The primary reaction products, polyunsaturated lipid hydroperoxides, are dioxygenated on the 1- or 5-carbon of the original pentadiene chain, depending on the regiospecificity of the enzyme. The best characterized lipoxygenase to date is the isozyme L-1 from soybean seed. It had been established in L-1, by site-specific mutations, that three of its His residues and Asn 694 are essential for binding of the iron and for enzyme activity (Steczko & Axelrod, 1992; Steczko et al., 1992). X-ray crystallographic studies by Boyington et al. (1993) indicate that the active center of L-1 contains iron coordinated with three His residues and the α-carboxyl of the C-terminal Ile. Studies by Minor et al. (1993) implicate the same residues plus Asn 694. These residues are highly conserved, occurring in virtually all 30 lipoxygenases tested, from both plant and animal sources.

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*Abbreviations:* L-1, soybean seed lipoxygenase isozyme L-1; L-3, soybean seed lipoxygenase isozyme L-3.

It is highly probable that all lipoxygenases catalyze essentially the same primary reaction although the site of oxygenation varies with the specific enzyme. For example, soybean lipoxygenase-1 produces the 13-hydroperoxide when linoleic acid is the substrate. Lipoxygenase-3, which accompanies L-1 in soybean seed and is the object of the present study, produces roughly equal amounts of the 9- and 13-hydroperoxides of the same substrate (Christopher et al., 1972).

The protein sequences of L-1 (839 residues) and L-3 (865 residues) are 81% identical (Shibata et al., 1988; Yenofsky et al., 1988). Despite the similarities in sequence and in primary mechanism, these enzymes exhibit some notable differences in behavior: (1) the regiospecificities are different as noted; (2) the pH optimum of L-1 is 9.0 compared to 7.0 for L-3; (3) the activity of L-3 shows an inverse dependence on the enzyme concentration, in contrast to L-1, which behaves conventionally and whose specific activity is independent of enzyme concentration; and (4) L-1 utilizes oxygen in a 1/1 stoichiometric relationship with the hydroperoxide formed, although it catalyzes the destruction of the hydroperoxide when oxygen is exhausted. L-3 catalyzes the destructive reaction even in the presence of oxygen.

The diverse regiospecificities exhibited by the human lipoxygenases have particular significance in health and disease because their products are the precursors of potent physiological effectors having markedly different functions (Yamamoto, 1992). Their natural substrate, eicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid, contains four pentadiene moieties and hence a number of putative hydroperoxidation sites. These enzymes are designated as 5-, 12-, or 15-Lox to reflect their specificities, which, however, are not absolute. The active centers of these enzymes, based on sequence comparisons and mutagenesis experiments, are virtually identical with those of L-1 as well as the other soybean isozymes and, indeed, with all lipoxygenases from plants (Ford-Hutchinson et al., 1994). Information concerning the structural differences between L-1 and L-3 should prove useful in the search for the structural basis for regiospecificity in the mammalian enzymes. Studies by Sloane et al. (1995) have shown that residues Ile 417 and Met 418 in human reticulocyte 15-Lox are in

a crucial region affecting the regiospecificity. Thus, the 15-Lox mutant, I417V-M418V, catalyzes oxygenation of arachidonic acid to give the 15- and 12-hydroperoxy products in a ratio of 1:20, in contrast to a ratio of 9:1 for the wild type. The authors surmise from these and other results that the effects may arise from changes in the fit of the substrate and the consequent orientation of its target diallylic region vis-à-vis the active center. However, full interpretation depends on a good visualization of the three-dimensional structure of the enzyme. In our goal to establish the basis of the positional specificities of the soybean lipoxygenases as well as the other differences and similarities noted above, it will be instructive to compare their well-resolved X-ray structures. To this end we have undertaken to obtain a high-resolution structure of L-3. We report here the crystallization and preliminary X-ray characterization of L-3.

L-3 was purified essentially by the procedure described previously (Axelrod et al., 1981). To show that the "L-3" fraction obtained from the final chromatographic separation was not contaminated with L-2 (which elutes close to L-3), a portion was subjected to limited trypsinolysis followed by SDS-PAGE. The separated polypeptides were electrophoretically transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue. Automated amino acid sequencing of the slowest-moving fragment resulted in the sequence as shown. Comparison with the corresponding regions of authentic L-2 (Shibata et al., 1988) and L-3 (Yenofsky et al., 1988) established unequivocally that the protein in question was L-3.

L-3 (333) E I F R T D G E Q **A** L K F P P P **K** V I Q

L-2 (344) E L F R T D G E Q V L K F P P P H V I Q

Found E I F R T D G E Q **A** L K F P P P **K** V I Q

Crystals were obtained using the sitting drop method on Hampton bridges (Hampton Research Co., Riverside, California) placed in wells of 24-well Linbro plastic culture dishes. Typically, 1 mL of the precipitant solution was placed in the well and 5 $\mu$L protein stock solution (1.5% in 0.02 M phosphate, pH 6.8) and 5 $\mu$L of precipitant solution were mixed in the cavity of the bridge. The dishes were covered with transparent tape and kept at 21°C. Conditions for crystallization were readily established. The precipitant solution contained 0.2 M sodium acetate buffer, pH 5.7, 16–20% polyethylene glycol (average $M_r$ = 3,400).

The first crystals, less than 0.1 mm in length, were smaller than desirable for preliminary characterization but were used for microseeding. Approximately 10 days after microseeding, larger pyramidal crystals were obtained and used in the X-ray studies. When 0.2 M citrate buffer, pH 5.6, was used, clusters of needles unsuited for diffraction studies resulted.

The crystallographic data were collected at the X-12C station at the National Synchrotron Light Source, Brookhaven National Laboratory, which was equipped with a Mar Research scanner. All data sets were processed, scaled, and postrefined using the HKL package (Denzo [Otwinowski, 1993a], Scalepack [Otwinowski, 1993b], and XdisplayF [Minor, 1993]). All partial reflections were added to determine the full intensity. The crystals diffracted to at least 3 Å resolution at room temperature (Fig. 1). The wavelength was 1.08 Å and the oscillation range was 2°. The $R$-factor was 6.7%, including all 43,087 reflections measured.

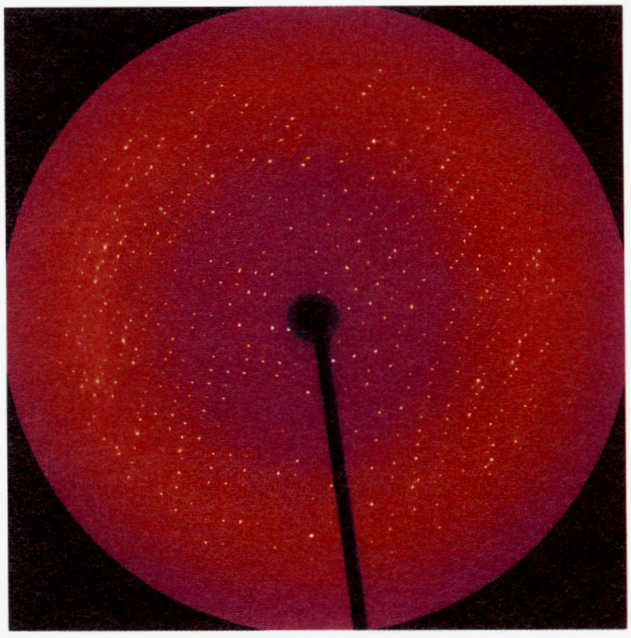

**Fig. 1.** Oscillation photograph from L-3 lipoxygenase recorded on X12C beam line at the National Synchrotron Light Source, Brookhaven National Laboratory. Wavelength was 1.08 Å and oscillation range was 2.0°. Crystal detector distance was 329 mm.

The data are summarized in Table 1. The crystals are monoclinic and belong to space group C2. They have the following cell constants: $a = 111.9$, $b = 136.4$, and $c = 61.6$ Å and $\beta = 95.7°$. The calculated Matthews's constant, $V_m$ = 2.48 Å/Da ($Z$ = 4), is reasonable, based on the compiled data of Matthews (1968). The corresponding value for L-1 is 2.41 Å$^3$/Da ($Z$ = 2) (Steczko et al., 1990).

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**Table 1.** *Summary of X-ray data collection*

| Resolution shell (Å) | Average intensity | No. of reflections | $R$-factor |
|---|---|---|---|
| 40.00–6.46 | 11,015 | 1,047 | 0.032 |
| 6.46–5.13 | 5,278 | 1,100 | 0.042 |
| 5.13–4.48 | 8,016 | 1,110 | 0.042 |
| 4.48–4.07 | 6,723 | 1,125 | 0.050 |
| 4.07–3.78 | 4,778 | 1,143 | 0.066 |
| 3.78–3.56 | 3,917 | 1,150 | 0.081 |
| 3.56–3.38 | 2,801 | 1,143 | 0.110 |
| 3.38–3.23 | 2,211 | 1,193 | 0.151 |
| 3.23–3.11 | 1,717 | 1,186 | 0.198 |
| 3.11–3.00 | 1,256 | 1,187 | 0.282 |
| All *hkl* | 4,667 | 11,384 | 0.067 |

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